



Divergence time analyses suggest a Miocene origin of the narrow Amazonian endemic rheophytic *Ceratolejeunea temnantha* (Spruce) Reiner-Drehwald (Porellales, Lejeuneaceae)

ADRIEL M. SIERRA^{1,2,3}, JULIA BECHTELER⁴, DOMINGOS CARDOSO⁵, CHARLES E. ZARTMAN^{1*} & JUAN CARLOS VILLARREAL^{2,3}

¹ Instituto Nacional de Pesquisas da Amazônia, Departamento de Biodiversidade, Av. André Araújo Aleixo, Caixa Postal 478, 69060001 Manaus, Brazil.

² Département de Biologie, Université Laval, Québec, Canada

³ Institut de Biologie Intégrative et des Systèmes (IBIS), Université Laval, Québec, Canada

⁴ Nees Institute for Biodiversity of Plants, University of Bonn, Bonn, Germany

⁵ Instituto de Biologia, Universidade Federal da Bahia (UFBA), Rua de Barão de Jeremoabo, s.n., Ondina, 40170-115, Salvador, Bahia, Brazil

*e-mail corresponding author: chaszartman@gmail.com

Abstract

The recent rediscovery of the rheophytic endemic *Ceratolejeunea temnantha* ~130 years after its original description, on the upper Rio Negro in the Brazilian Amazon, has enabled the assessment of its enigmatic phylogenetic position, estimates of its divergence time, and updates on its distribution and potential habitat threats. Phylogenetic analyses strongly supported its placement in the genus *Ceratolejeunea* in a geographically disparate clade including a Madagascar endemic *C. saroltae* and two Neotropical taxa, *C. confusa* and *C. caducifolia*. Divergence time estimates date the clade's stem age to the late Miocene (8.92 [HPD: 12.39–6.04] Ma) offering further evidence that the evolution of rheophytes in northern South America is correlated with the expansion of cryptogams into novel ecological niches promoted by dramatic landscape changes during the Miocene. Major geomorphological and hydrological transformations contributing to such diversification are most likely the changing dynamics of the inundated mega lake system to the establishment of the Amazon River due to the Andean orogeny and the subsequent cessation of marine influences in the north-western portion of the Basin. Until recently, this rheophyte of seasonally inundated black-water forests was only known from its type collection from the Rio Negro near São Gabriel da Cachoeira (Brazil) as described by Richard Spruce in 1884. These new collections extend the distribution of this rare narrow endemic to the middle Rio Uaupés, a tributary of the upper Rio Negro near the Columbian border.

Keywords: Amazon, black-water flooded forests, landscape evolution, liverworts, Igapó, Lejeuneaceae, Pebas mega-wetland

Introduction

Neotenic and atypical morphologies common among the Lejeuneaceae have traditionally hampered the understanding of phylogenetic relationships in this family, thus necessitating the use of molecular data to verify infra-familial relationships (Gradstein *et al.* 2006, 2011, Wilson *et al.* 2007a, Heinrichs *et al.* 2012, 2013, Yu *et al.* 2014). For example, rheophytic Lejeuneaceae display extreme cases of morphological (e.g., creeping rhizomes and thick stems) and phenological adaptations (e.g., high sexual expression) most likely attributable to adaptations imposed by the mechanical and environmental stresses of the ecological demands of surviving in aquatic and seasonally flooded habitats (Gradstein *et al.* 2011, Thiers 1984, 1988). Rheophytism has indeed evolved independently among liverworts, mosses and hornworts (Gradstein *et al.* 2001, Shevock *et al.* 2017). In Neotropical liverworts, the Lejeuneaceae presents examples of convergent evolution in riparian habitats, and such cases are best illustrated in taxa occurring in the northern Andes and the Amazon basin (Gradstein *et al.* 2001, Reiner-Drehwald & Weis 2001, Gradstein & Costa 2003, Gradstein *et al.* 2011, Reiner-Drehwald 2011, Bastos 2017, Heinrichs *et al.* 2012, 2013).

For example, an extreme case of rheophytic adaptation in Lejeuneaceae is observed in *Colura irrorata* (Spruce 1884) Heinrichs, Y. Yu, Schäf.-Verw. & Pócs (2012) which has long received attention due to its unusual morphology

(Spruce 1884, Thiers 1984, Gradstein *et al.* 2004). This species is presently known from two localities (Topo river in Ecuador and Numpatakaima river in Peru), and its restricted distribution might be associated to the geological granite intrusions of Jurassic age substrates of the Andes (Gradstein *et al.* 2004, Gradstein & Benítez 2014). A molecular study based on two plastid and one nuclear marker nested this odd rheophytic liverwort in *Colura* (Dumort. 1831) Dumort. (1835) as sister to the widespread *Colura calyptrifolia* (Hook. 1813) Dumort. (1835). However, both species display contrasting morphologies and diverged relatively recently (~7–30 Ma; Laenen *et al.* 2014) suggesting rapid morphological rearrangement due to selection from ecologically divergent habitats (Heinrichs *et al.* 2012).

Another rheophytic species from the northern Andes, *Lejeunea topoensis* Gradst. & M.E. Reiner (2007), likewise, shows a relatively recent divergence time of ~10 Ma (Heinrichs *et al.* 2016). However, considering that only 57 out of more than the 400 accepted species of *Lejeunea* Libert (1820) (Söderström *et al.* 2016, Heinrichs *et al.* 2016) have been sampled, it could be that this age estimate does not accurately reflect its evolutionary history. Despite information on the phylogenetic placement and divergence times for these taxa, few studies have yet to directly assess the potential ecological and evolutionary causes of rheophytism (Heinrichs *et al.* 2012, Heinrichs *et al.* 2013, Gradstein *et al.* 2011). These age estimates, which range from the Oligocene to the early Pliocene, indeed correspond to an epoch of major geological change in northern South America (Shepard *et al.* 2010), which has duly been independently correlated with accelerated diversification rates of disparate lineages of plants and animals in this region (Antonelli & Sanmartín 2011, Hoorn *et al.* 2010). However, estimates of age diversification of liverwort species in northern South America, particularly those of extreme habitats, are relatively scarce calling for studies to address the role of landscape change on the diversification of cryptic yet ecologically important plants groups, such as the Lejeuneaceae, in the Amazon.

The genus *Ceratolejeunea* (Spruce 1884) J.B. Jack & Steph. (1892) has *ca.* 40 species worldwide, and species occur as epiphytes, epiphylls and rheophytes in tropical forests (Dauphin 2003, Reiner-Drewald 2011). A recent dated phylogeny of *Ceratolejeunea* involving twenty of the forty accepted species across the Neo- and Paleotropics, provided an initial framework for the natural classification of the genus (Scheben *et al.* 2016). Furthermore, the study points to a Neotropical origin of the genus with recurrent dispersal events and subsequent expansions to other continents from the late Oligocene to Pleistocene (Scheben *et al.* 2016). However, to get to know more about the evolutionary history of *Ceratolejeunea* the investigation of further taxa is needed for a better understanding of the diversification processes in this ecologically important liverwort genus.

In *Hepaticae Amazonicae and Andinae*, Richard Spruce described the basionym *Lejeunea temnantha* Spruce (1884) along with other rheophytic Lejeuneaceae under *Lejeunea* subgen. *Potamolejeunea* Spruce (1884). Recently, this species was transferred to *Ceratolejeunea*, based on its dark color, brown middle lamella of the leaf cell walls, presence of ocelli, pycnolejeuneoid-innovations, and perianths with 4–5 keels extending into short horns (Reiner-Drehwald 2011). However, this species was, until recently, only known from the type collection (Spruce 1884) along seasonally inundated banks of the upper Rio Negro: the largest black-water tributary of the Amazon Basin. In December of 2017, during a collecting excursion conducted by several of the co-authors multiple populations of this species were relocated along the banks of the Rio Negro and Rio Uaupés near the town of São Gabriel da Cachoeira located west of Manaus, the capital of the Brazilian State of Amazonas (Figure 1).

The re-discovery of *Ceratolejeunea temnantha* offers the opportunity, using an available dated phylogeny, to provide a temporal and phylogenetic framework for the evolution of this rheophyte as well as other rare species of similar habitats in the Amazon Basin: black-water forests (referred to as *Igapó* in local parlance). Specifically, we aim to: i) elucidate the phylogenetic relationship of the only known rheophytic species in the genus *Ceratolejeunea*, ii) estimate the age of divergence of the species in the context of Amazonian ecological and species diversity, and iii) update the current distribution of the species.

Material and Methods

Taxon sampling

To determine the phylogenetic relationship of the only rheophytic species in the genus *Ceratolejeunea*, sequences from two accessions of *C. temnantha* were generated (Table 1). Furthermore, we include sixty-one accessions downloaded from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) representing twenty-three species in our molecular dataset. The species *Luteolejeunea herzogii* (Buchloh 1961) Piippo (1986) was used as the outgroup taxon, based on previous studies (Wilson *et al.* 2007, Scheben *et al.* 2016). Species names, voucher information and GenBank accession numbers for all sequences are listed in Supplementary Table 1.



FIGURE 1. Population distribution of *Ceratolejeunea temnantha*: around the type locality on the Upper Rio Negro near Sao Gabriel da Cachoeira City (Diamond ◆) and newly recorded population along the Rio Uaupés in Brazil, Amazonas (Triangle ▲).

TABLE 1. Sequences used in this study, including species names, voucher information and GenBank accession numbers.

Species	Voucher information	<i>rbcL</i>	<i>trnL</i>	ITS
<i>Ceratolejeunea temnantha</i> (Spruce) M.E.Reiner	Brazil. Amazonas. Sierra A.M. 5062	MK061421	MK061417	MK061419
<i>Ceratolejeunea temnantha</i> (Spruce) M.E.Reiner	Brazil. Amazonas. Sierra A.M. 5083	MK061422	MK061418	MK061420

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from fresh plant tissue using DNeasy Plant Mini Kits (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was carried out to amplify three molecular markers: the plastid regions *rbcL* and *trnL-F*, and the nuclear ribosomal internal transcribed spacer (ITS) region, nrITS1–5.8S-ITS2 (Hartmann *et al.* 2006, Gradstein *et al.* 2006). Sequencing primers were the same as those used for the PCR reactions. Alignment for each nuclear and plastid marker was executed separately using Geneious 5.6.6 multiple alignment with 65% (Biomatters Ltd, Auckland, New Zealand), using default settings and subsequent manual verification. The three molecular markers were concatenated consisting of a matrix of 2,843 nucleotides, and upon exclusion of ambiguous sites a total of 2,498 characters were then used in downstream phylogenetic analyses.

Phylogenetic analyses

Maximum parsimony (MP) analyses were carried out with PAUP* 4.0a146 (Swofford 2000). MP heuristic searches were conducted with the following options: heuristic searches mode 1,000 random-addition-sequence replicates, tree bisection-reconnection (TBR) branch swapping with multiple trees saved. All characters were treated as equally weighted and unordered.

The software Partition Finder (Lanfear *et al.* 2016) was used to select the best partition schemes and evolution models for the three molecular markers. For *rbcL* we selected the best partition scheme by codons, which indicate the GTR+I+Γ model for the 1st and 2nd positions and the GTR+Γ for the 3rd position. For the *trnL-F* including the *trnL* intron and the *trnL-F* spacer, as well for the ITS region the GTR+I+Γ was specified as the best model. Maximum

Likelihood (ML) analyses were performed with the program RAxML-Blackbox using 1000 bootstrap pseudoreplicates (Stamatakis 2016) under the Gama model of rate heterogeneity. A 50% majority rule consensus tree was computed from the 1,000 bootstrapping pseudoreplicates with PAUP (Swofford 2000). The result was visualized in FigTree v1.4.3 (Rambaut 2014), and bootstrap values (MLB) ≥ 70 were regarded as moderate and > 80 as good support (Erixon *et al.* 2003).

Bayesian inference (BI) was performed using MrBayes v.3.2.6 (Ronquist & Huelsenbeck 2003) and computations using the Cipres Science Gateway (Miller *et al.* 2010). For the BI analysis, the models of evolution selected by Partition finder (Lanfear *et al.* 2016) were defined for each partition. Default priors of model parameters were also defined for each partition. Two parallel Markov Chain Monte Carlo (MCMC) runs together adding up to ten million generations were conducted each run containing eight chains with default priors on most parameters. Trees and estimated parameter values were sampled every 1,000 generations, thus obtaining a total of 10,000 samples from which the first 1,000 (10%) were discarded as burn-in. Burn-in and convergence of runs were checked using Tracer 1.6 (Rambaut *et al.* 2014). A majority-rule consensus tree was computed to calculate the Bayesian posterior probability (BPP), which values of BPP ≥ 0.95 were considered as significant.

Molecular clock dating

We used an internal fossil calibration (Heinrichs *et al.* 2015) in combination with a secondary calibration on the root of the tree (Feldberg *et al.* 2014, Scheben *et al.* 2016) to evaluate the divergence time within *Ceratolejeunea*. The fossil *Ceratolejeunea sublaetefusca* Heinrichs, Pócs & Schäf.-Verw. (2015) (Heinrichs *et al.* 2015) is a Miocene Mexican amber fossil contemporary to Dominican amber fossils (Solorzano Kraemer 2007) dated to 15–20 Ma (Iturralde-Vinent & MacPhee 1996). We placed the fossil on the stem of the clade containing the extant *C. laetefusca* (Austin 1876) R.M. Schust. (1956) which is morphologically similar to the fossil (Heinrichs *et al.* 2015). As suggested by Scheben *et al.* (2016), we used a lognormal distribution (offset: 14.5, mean: 1, SD: 1; 95% interval: 15–28.6 Ma). The divergence between *Luteolejeunea* Piippo (1986) and *Ceratolejeunea* is dated to 36 Ma (SD: 5) (Feldberg *et al.* 2014) using a normal distribution following Scheben *et al.* (2016). All other priors were left as default values.

We selected thirty-two accessions which represent a total of twenty *Ceratolejeunea* species and the out-group, considering their provenance and phylogenetic position. We included the species *Ceratolejeunea minuta* Dauphin (2003), *C. desciscens* (Sande Lac. 1856) Schiffn. (1893), *C. confusa* R.M.Schust. (1956), and *C. coarina* (Gottsche 1845) Schiffn. (1893), with 1–2 markers missing, since small proportion of missing data would not affect the divergence time estimates (Zheng & Wiens 2015).

Divergence times were estimated using BEAST v1.8.4 (Drummond *et al.* 2012) and a pure-birth Yule prior and the previous described substitution model with unlinked data partition for the three markers. The analyses used an uncorrelated log-normal relaxed clock model (Drummond *et al.* 2006). Markov chain Monte Carlo (MCMC) was run for 650 million generations with parameters sampled every 10,000 generations.

The convergence point was estimated by examining the three independent log files in Tracer 1.6 (Rambaut *et al.* 2014) to confirm that separate analyses converged on the same result. Effective sample size values > 200 were regarded as good sampling, indicating that the parameter space had been sampled sufficiently for valid parameter estimation. Runs converged around 50 million generations. The initial 10% of trees were discarded as burn-in and a maximum clade credibility tree with mean node heights was constructed from the remaining trees with TreeAnnotator 1.8.2 (part of the BEAST package) and visualized with Figtree v1.4.3 (Rambaut 2014). An additional analysis with an empty alignment was run to test the influence of the priors on posterior distributions. We report mean ages and the 95% high posterior density (HPD) values.

Morphological and ecological observations

Fresh samples of *Ceratolejeunea temnantha* were studied using a light microscope equipped with a digital camera. Oil bodies were observed from selected samples of *Ceratolejeunea temnantha*. Geographical and ecological data were gathered based on field observations where populations were relocated along the Rio Negro and Rio Uaupés.

Results

All phylogenetic analyses (MP, ML and BI) show congruent topologies which only differed in the degree of support for some clades. From the concatenated data analysed (2,498), 1,868 characters were constant (proportion=0.74), and 216 variable characters were parsimony-uninformative while 414 were parsimony-informative characters (Table 2).



Figure 2

FIGURE 2. Majority rule consensus tree from Bayesian inference of the genus *Ceratolejeunea* based on three nuclear and plastid markers. ML bootstrap values ≥ 50 and Bayesian posterior probability values ≥ 0.95 are depicted on branches. The clade of *Ceratolejeunea temnantha* is highlighted in grey.

TABLE 2. Summary of phylogenetic constant and parsimony informative characters for the concatenated alignment of the three markers.

Markers	Characters	Constant	Proportion	Parsimony-uninformative	Parsimony informative
<i>rbcL</i>	921	841	0.91	40	40
<i>trnL</i>	469	384	0.81	31	54
ITS	1108	643	0.58	145	320
Complete	2498	1868	0.74	216	414

Ceratolejeunea temnantha is firmly placed within the genus *Ceratolejeunea* (Figure 2). Both accessions of *C. temnantha* form part of a clade (MP=100, MLB=100, BPP=1.00) including the Neotropical species *C. caducifolia* (Spruce 1884) Steph. (1913) and *C. confusa*, and the Madagascar endemic *Ceratolejeunea saroltae* Pócs (2011). The relationship among these species within the well supported clade was not supported by ML and BI analyses as they were recovered in a polytomy (Figure 2). The result of MP placed the accessions of *C. temnantha* as sister to *C. saroltae*, *C. confusa* and *C. caducifolia* with total support (MP=100) (Supplementary Figure 1). In all analyses, the clade with *C. temnantha* is found sister to a clade consisting of *C. globulifera* Herzog (1942), *C. grandiloba* J.B. Jack & Steph. (1892), and *C. desciscens*.

Using both calibrations, the crown age of the genus *Ceratolejeunea* is 33.94 [95% HPD: 26.37–42.23] Ma (Table 3). The most recent ancestor of the rheophytic species (stem age) dates to 8.92 [6.04–12.39] Ma, and the crown age to 0.21 Ma [95% HPD: 0.004–0.62] Ma (Figure 3). Morphological observations (Figure 4) and ecological notes for *Ceratolejeunea temnantha* are given in the description section below.

TABLE 3. Divergence time estimates under a relaxed clock for nodes of interest in *Ceratolejeunea*. Age estimates are in millions of years before present (Ma). The 95% highest posterior density (HPD) intervals (in square brackets) and Bayesian posterior probability support values are given for each node of interest (BPP) Node numbers correspond to Figure 3.

Node	Estimated divergence time in millions of years ago/before present with 95% HPD
1 (BPP=1.0)	42.21[31.02, 56.41]
2 (BPP=1.0)	33.94 [26.37, 42.23]
3 (BPP=0.99)	24.23 [19.19, 31.17]
4 (BPP=1.0)	15.54 [11.50, 20.73]
5 (BPP=0.99)	20.37 [16.46, 26.27]
6 (BPP=0.87)	16.85 [14.63, 21.46]
7 (BPP=0.66)	18.69 [14.73, 24.45]
8 (BPP=1.0)	8.64 [6.40, 11.57]
9 (BPP=1.0)	8.92 [6.04, 12.39]
10 (BPP=1.0)	0.21 [0.004–0.62]

Discussion

Phylogenetic affinities of Ceratolejeunea temnantha

Traditionally the genus *Ceratolejeunea* has been classified in two subgenera (Dauphin 2003, Schuster 1978, 1956). *Ceratolejeunea* subgen. *Ceratophora* R.M. Schust. (1956) is characterized by entire underleaves and boubous perianth horns, and *C.* subgen. *Ceratolejeunea* R.M. Schust. (1978) is characterized by bifid underleaves and variable perianth horns. However, these subgeneric classifications are not monophyletic (Scheben *et al.* 2016). *Ceratolejeunea temnantha* was placed in *C.* subgen. *Ceratolejeunea* according to its morphology (Reiner-Drehwald 2011), and results herein resolved it with strong support in a clade sister to some taxa traditionally considered in *C.* subgenus *Ceratophora*.

Our results indicate that *Ceratolejeunea temnantha* is related to the Neotropical species *C. caducifolia*, and *C. confusa* as well as the Madagascar endemic *C. saroltae*. Interestingly, these species have perianths with indistinct or short horns, also found in *C. minuta*, resolved in a separate monophyletic clade. *Ceratolejeunea temnantha* morphologically resembles to the epiphytic *C. confusa* which occurs sympatrically in Rio Negro. The abundance of

fertile plants is only observed in two of the species in this clade: *C. temnantha* and *C. saroltae*. However, *C. saroltae* occurs as an epiphyll and an epiphyte in montane forests of Madagascar (Pócs 2011), in contrast to *C. temnantha*, which is restricted to Amazonian lowland black-water rivers. Nevertheless, the phylogenetic relationships within the clade including *C. caducifolia*, *C. confusa*, *C. temnantha* and *C. saroltae* are not resolved with our dataset.

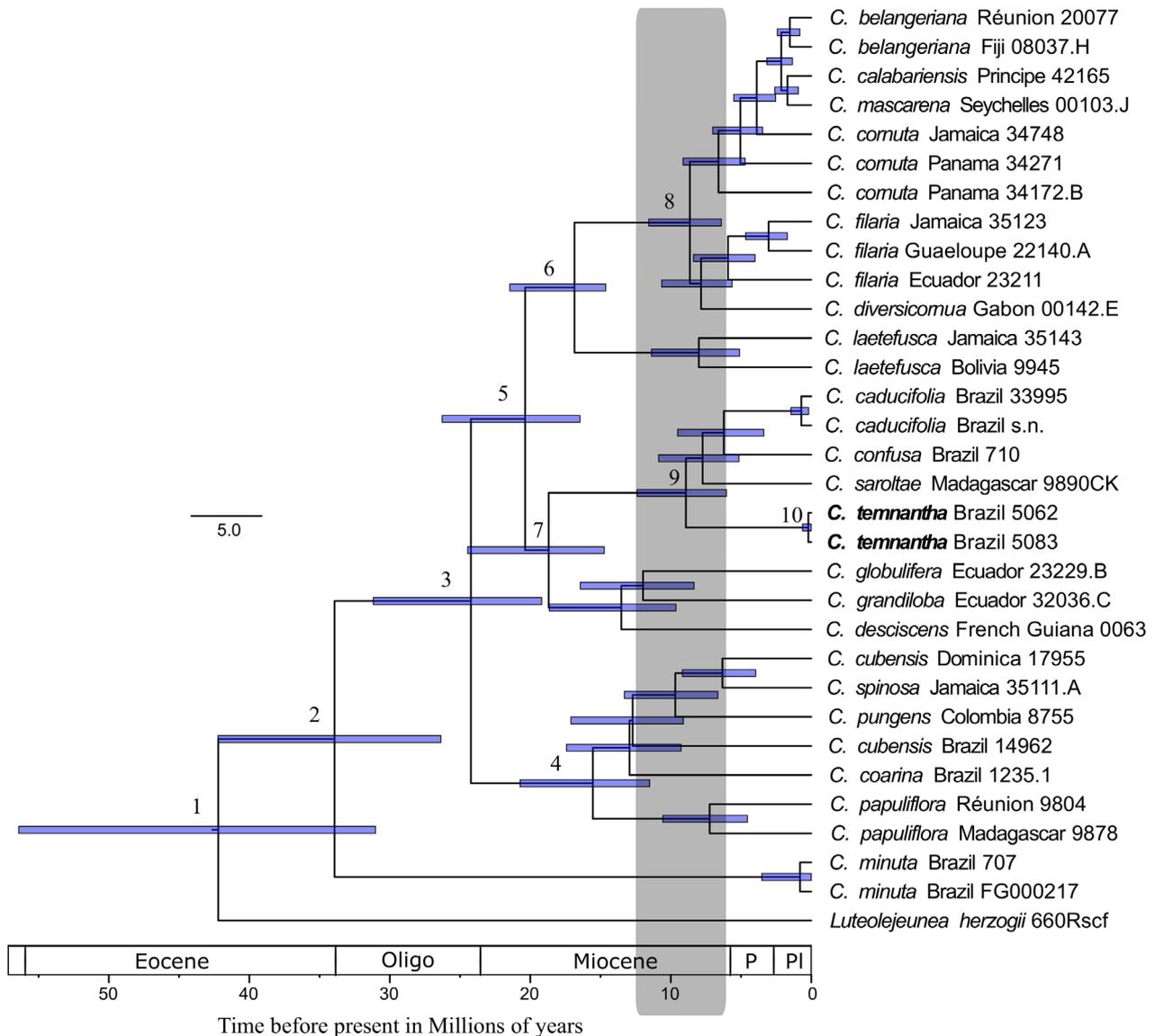


FIGURE 3. Chronogram of the divergence time analysis of the genus *Ceratolejeunea* inferred from nuclear and plastid DNA, under a relaxed clock model calibrated with a fossil and secondary calibration. Highlighted in grey is the 95% highest posterior density (HPD) interval of *Ceratolejeunea temnantha*. Bars at nodes indicate 95% HPD intervals around node ages.

Divergence time of Ceratolejeunea temnantha and evolution of rheophytism in Lejeuneaceae

Our results (Table 3) corroborate with previous divergence time estimates for the genus *Ceratolejeunea* as originating during the Paleogene, and most diversification occurring during the Oligocene and Miocene (Scheben *et al.* 2016). The divergence time of the most recent ancestor of *Ceratolejeunea temnantha* was dated at 8.92 [HPD: 6.04–12.39] Ma coinciding with the end of the most intense peaks of the northern Andean orogeny: the late-middle Miocene [~12 Ma] to the early Pliocene [~4.5 Ma] (Hoorn *et al.* 2017). During the Paleogene [65–34 Ma], plate tectonic changes caused the uplift of the central and northern Andes. The northern Andes cordillera results from two orogeny events, one by the late Oligocene to early Miocene [~23 Ma], and a second during the late middle Miocene to early Pliocene (Shepard *et al.* 2010; Hoorn *et al.* 2010). Parallel to the uplift of the Andes, the formation of a vast expanse of wetlands, known as the Pebas mega-wetland system (Hoorn *et al.* 2010), consisting of shallow lakes and swamps developed across western Amazonia which experienced two major marine influx intervals during the Miocene (Jaramillo *et al.* 2017).

The uplift of the central and northern Andes dramatically modified the topography and, subsequently, the hydrological dynamics of the Amazon Basin. These changes included reversal drainages of the Amazon River as well as the retraction of the Pebas mega-wetland in western Amazonia (Shepard *et al.* 2010). Such geological phenomena drove rapid diversification in western Amazonia among disparate plant and animal lineages that contributed significantly to the present mega diversity of the Amazon Basin (Antonelli & Sanmartín 2011, Hoorn *et al.* 2010). Divergence time estimates for *C. temnantha* indeed corroborate with the notion that this rheophytic bryophyte evolved in a period when hydrological conditions changed dramatically and when salinity levels, an aquatic environment uninhabited by extant bryophytes, would have fallen for the final time (Jaramillo *et al.* 2017). A dated phylogeny of two other rheophytic bryophytes restricted to the northern Andes (Heinrichs *et al.* 2012, 2013) also correlates the evolution of rheophytism in bryophytes with massive landscape changes during the Miocene. Although, the precise geological and evolutionary processes contributing to the origin of neotropical biodiversity is still incipient, there is mounting evidence pointing to the Amazonian basin as the cradle of diversification for this entire region (Antonelli *et al.* 2018).

Indeed, species with restricted distributions across northern South America could be used to address the assembly of diversity across evolutionary time scales in this biogeographical region. Further investigation including other rheophytic taxa from phylogenetically independent lineages would be necessary to correlate the advent of rheophytism with the uplift of the Andes and subsequent drainage changes imposed on the Amazon Basin during the Miocene [e.g., *Cololejeunea stoteleriana* Gradst., Ilk.-Borg. & Vanderp. (2011), *Schusterolejeunea inundata* (Spruce 1884) Grolle (1980), *Cephalantholejeunea temnanthoides* R.M.Schust. (1980), *Cheilolejeunea polystachya* (Spruce 1884) Gradst. & Ilk.-Borg. (2009)]

Rediscovery of the rheophytic endemic *C. temnantha* and its conservation status

Ceratolejeunea temnantha represents another rheophytic species rediscovered ~130 years after its original description (Spruce 1885; Bastos 2017, Gradstein *et al.* 2004, Gradstein & Benitez 2014). Recent studies point out that several endemic species from the upper Rio Negro are only locally abundant but with a restricted distribution (Gradstein *et al.* 2001, Pócs 2002). This may explain why such species have rarely been collected, as the upper Rio Negro region remains nearly unexplored.

We collected *Ceratolejeunea temnantha* in seasonally inundated black-water forest habitats near São Gabriel da Cachoeira, where Spruce might have gathered the type collection. It is locally abundant forming large mats on rocks, lower portions of tree trunks, twigs and roots in running water (seasonal habitats) usually growing with *Schusterolejeunea inundata*. Three new populations were found along the Rio Uaupés in this type of environment (Figure 4 A–B), one of which is located approximately 150 km from the Colombian border (Figure 1). Efforts should be taken to search for this and other rare endemics of the upper Rio Negro along the same tributaries of the Colombian Amazon.

C. temnantha covers entire roots, tree trunks up to 2 meters high and large area of rocks along the rivers (Rio Negro and Rio Uaupés). It occurs in rather open areas along river banks and depressions which may be inundated for months on an annual basis (Figure 4 A–B). Curiously, it occurs patchily in this region perhaps suggesting that meteorological and hydrological changes for the Amazon Basin predicted by climate change models (Malhi *et al.*, 2009) place *C. temnantha* as vulnerable for its conservation status.

Description

***Ceratolejeunea temnantha* (Spruce) Reiner-Drehwald (Figure 4)**

Lejeunea temnantha was described by Spruce, and subsequently illustrated and given the new combination *Ceratolejeunea temnantha* by Reiner-Drehwald (2011). It is characterized by its dark to blackish colour in both fresh and dry condition, cell wall middle lamella brown, presence of 1–2 basal small ocelli in leaf lobes, highly fertile with gynoecia with pycnolejeuneoid-innovations, perianths with (4–) 5 keels, with the ventral and lateral keels +/--extended into short horns. Oil bodies 1–5 per leaf cell of the *Calypogeia*-type: elliptical to spherical, granular (Figure 4 C–D).

Ecology and Distribution: The species *Ceratolejeunea temnantha* is restricted to seasonally flooded environments along the Brazilian portion of the upper Rio Negro (São Gabriel da Cachoeira and Rio Uaupés; Figure 1), where it grows forming large mats on trees trunks and roots, rocks, and soil on flooded habitats (Figure 4 A–B). It was found growing with *Schusterolejeunea inundata*, *Vitalianthus aphanellus* (Spruce 1884) Bechteler, G.E. Lee, Schäf.-Verw. & Heinrichs (2016), and *Cheilolejeunea polystachya*.

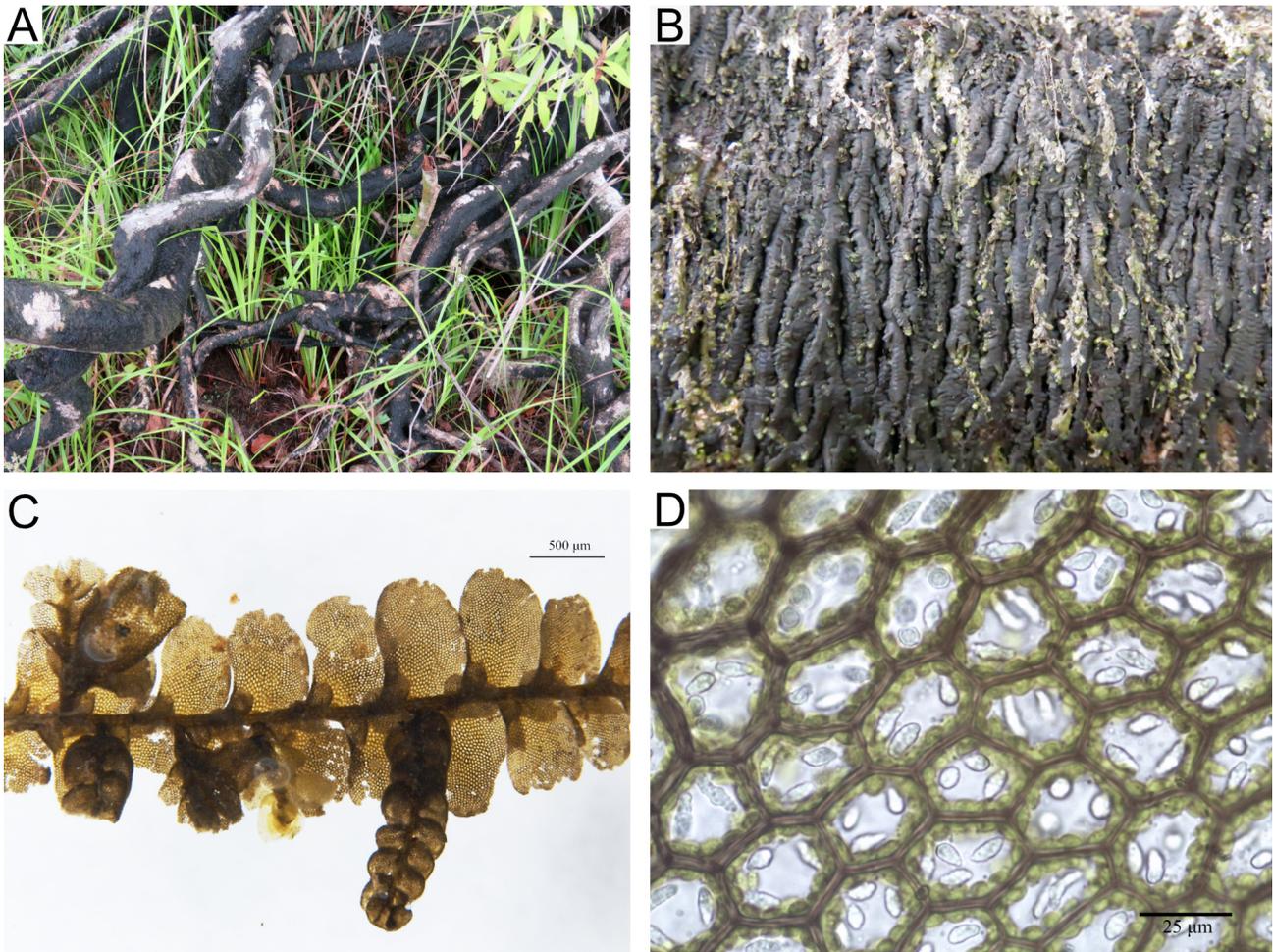


FIGURE 4. *Ceratolejeunea temnantha*: A) Rheophytic habit along the Rio Uaupés. Growing on exposed root on seasonal inundated habitat. B) Rheophytic habit along the Rio Uaupés. Growing with *Schusterolejeunea inundata* on tree trunk on seasonal inundated habitat. C) Fertile shoot with antheridial branches and perianth. D) Oil bodies: *Calypogeia*-type.

Specimens examined: BRAZIL. Amazonas: São Gabriel da Cachoeira, Rio Negro, 20 km downstream from the city São Gabriel da Cachoeira, 00°10'47.5" S 67°01'25.0" W. 16 December 2017, *Sierra 4701, 4703, 4704* (INPA). Rio Negro, downstream from the São Gabriel da Cachoeira city, Cariuari Island, 00°11'45.8" S 67°00'10.9" W. 17 December 2017, *Sierra 4715, 4716, 4718, 4720, 4723, 4724* (INPA). Rio Uaupés, proximo a comunidade de São Pedro, 00°06'43.9" N 67°39'19.1" W, 19 December 2017, *Sierra 4800* (INPA). Rio Uaupés, Sitio São Paulo, 21 December 2017, *Sierra 5015* (INPA, QFA), *5019, 5021, 5024, 5051, 5054, 5055, 5057, 5058, 5059* (INPA), *5056, 5062* (INPA, QFA). Rio Uaupés, Comunidade de Ananas, 22 December 2017, *Sierra 5083* (INPA), *5084* (INPA, QFA).

Acknowledgements

AMS, CEZ and DC thank the *Fundação Nacional do Índio* (FUNAI) and the *Federação das Organizações do Rio Negro* (FOIRN) for authorization to collect, and for offering logistic support during the excursions. CEZ acknowledges FAPEAM (CHAMADA N. 001/2015-CNPQ/MCTIC/FAP/PROTAX) of the State of Amazonas and the Federal Brazilian Government for financial support. The authors also thank *Instituto Chico Mendes de Biodiversidade* (ICMBio) for authorizing collecting permits and providing logistic support. AMS is grateful for financial support for graduate study by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES) Finance Code 001, and from the Scholarship Emerging Leaders of the America Program (ELAP), from the Government of Canada. DC acknowledges the CNPq Research Productivity Fellowship (306736/2015-2), Prêmio CAPES de Teses (23038.009148/2013-19), and FAPESB (APP0037/2016) for financial support.

Literature cited

- Antonelli, A., Zizka, A., Carvalho, F.A., Scharn, R., Bacon, C.D., Silvestro, D. & Condamine, F.L. (2018) Amazonia is the primary source of neotropical biodiversity. *Proceeding of the National Academy of Sciences* 115: 6034–6039.
<https://doi.org/10.1073/pnas.1713819115>
- Antonelli, A. & Sanmartín, I. (2011) Why are there so many plant species in the Neotropics? *Taxon* 60: 403–414.
- Austin, C.F. (1876) Notes on Hepaticology. *Botanical Gazette* 1: 35–36.
- Bastos, C.J.P. (2017) O gênero *Cheilolejeunea* (Spruce) Steph. (Lejeuneaceae, Marchantiophyta) nas Américas. *Pesquisas, Botânica* 70: 5–78.
- Bechteler, J., Lee, G.E., Schäfer-Verwimp, A., Pócs, T., Peralta, D.F., Renner, M.A., Schneider, H. & Heinrichs, J. (2016) Towards a monophyletic classification of Lejeuneaceae IV: reinstatement of *Allorgella*, transfer of *Microlejeunea aphanella* to *Vitalianthus* and refinements of the subtribal classification. *Plant Systematics and Evolution* 302: 187–201.
<https://doi.org/10.11646/phytotaxa.280.3.4>
- Buchloh, G. (1961) Einige Species novae und Neufunde von Moosen aus den Anden von Peru. *Nova Hedwigia* 3: 507–517.
- Dauphin, G. (2003) *Ceratolejeunea*. *Flora Neotropica monograph* 90: 1–87.
- Drummond, A.J., Ho., S.Y., Phillips, M.J. & Rambaut, A. (2006) Relaxed phylogenetics and dating with confidence. *PLoS Biology* 4, e88.
<https://doi.org/10.1371/journal.pbio.0040088>
- Drummond, A.J., Suchard, M.A., Xie, D. & Rambaut, A. (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* 29: 1969–1973.
<https://doi.org/10.1093/molbev/mss075>
- Dumortier, B.C.J. (1831) *Sylloge Jungermannidearum Europae indigenarum: earum genera et species systematice complectens*. Casterman, Tournay, 100 pp.
<https://doi.org/10.5962/bhl.title.22343>
- Dumortier, B.C.J. (1835) Recueil d'Observations sur les Jungermanniacées. *Blanquart, Tournay* 27 pp.
<https://doi.org/10.5962/bhl.title.731>
- Erixon, P., Svennblad, B., Britton, T. & Oxelman, B. (2003) Reliability of Bayesian posterior probabilities and bootstrap frequencies in phylogenetics. *Systematic Biology* 52: 665–673.
<https://doi.org/10.1080/10635150390235485>
- Feldberg, K., Schneider, H., Stadler, T., Schäfer-Verwimp, A., Schmidt, A.R. & Heinrichs, J. (2014) Epiphytic leafy liverworts diversified in angiosperm-dominated forests. *Scientific Reports* 4: 5974.
- Gradstein, S.R. & Costa D.P. (2003) The Hepaticae and Anthocerotae of Brazil. *Memoirs of the New York Botanical Garden* 88: 1–673.
- Gradstein, S.R., Churchill, S.P. & Salazar, N.A. (2001) Guide to the Bryophytes of Tropical America. *Memoirs of the New York Botanical Garden* 86: 1–577.
- Gradstein, S.R. & Ilkiu-Borges, A.L. (2009) Guide to the Plants of Central French Guiana. Part IV. Liverworts and Hornworts. *Memoirs of the New York Botanical Garden* 76 (4): 1–140.
- Gradstein, S.R., Wilson, R., Ilkiu-Borges A.L. & Heinrichs, J. (2006) Phylogenetic relationships and neotenic evolution of *Metzgeriopsis* (Lejeuneaceae) based on chloroplast DNA sequences and morphology. *Botanical Journal of the Linnean Society* 151: 293–308.
<https://doi.org/10.1111/j.1095-8339.2006.00531.x>
- Gradstein, S.R. & Reiner-Drehwald, M.E. (2007) The status of *Neopotamolejeunea* (Lejeuneaceae) and description of a new species from Ecuador and Southern Brazil. *Systematic Botany* 32: 487–492.
<https://doi.org/10.1600/036364407782250571>
- Gradstein, S.R., Reiner-Drehwald, M.E. & Schneider, H. (2003) A phylogenetic analysis of the genera of Lejeuneaceae (Hepaticae). *Botanical Journal of the Linnean Society* 143: 391–410.
<https://doi.org/10.1111/j.1095-8339.2003.00235.x>
- Gradstein, S.R., Reiner-Drehwald, M.E. & Jost, L. (2004) The systematic position and distribution of *Myriocolea irrorata* (Lejeuneaceae), an endangered liverwort of the Ecuadorian Andes. *Journal of the Hattori Botanical Laboratory* 95: 235–248.
- Gradstein, S.R., Ilkiu-Borges, A.L. & Vanderpoorten, A. (2011) Habitat specialization triggers the evolution of unusual morphologies—the case of *Cololejeunea stotleriana* sp. nov. from Ecuador. *The Bryologist* 114: 9–22.
<https://doi.org/10.1639/0007-2745-114.1.9>
- Gradstein, R. & Benitez, A. (2014) A second locality for the critically endangered *Colura irrorata* (Lejeuneaceae) in the Ecuadorian Andes. *Journal of Bryology* 36: 151–155.
<https://doi.org/10.1179/1743282013Y.0000000088>

- Gottsche, C.M., Lindenberg, J.B.W. & Nees von Esenbeck, C.G. (1845) Synopsis Hepaticarum, fasc. 3. *Meissnerianis*, Hamburg.
- Grolle, R. (1980) *Schusterolejeunea* Grolle nom. nov. statt *Cladocolea* Schust. 1963, non van Tieghem 1895. *Journal of Bryology* 11: 105–106.
<https://doi.org/10.1179/jbr.1980.11.1.105>
- Hartmann, F.A., Wilson, R., Gradstein, S.R., Schneider, H. & Heinrichs, J. (2006) Testing hypotheses on species delimitations and disjunctions in the liverwort *Bryopteris* (Jungermanniopsida: Lejeuneaceae). *International Journal of Plant Sciences* 167: 1205–1214.
<https://doi.org/10.1086/508023>
- Heinrichs, J., Dong, S., Yu, Y., Schäfer-Verwimp, A., Pócs, T., Feldberg, K., Hentschel, J., Schmidt, A. & Schneider, H. (2012) A 150-year mystery solved: Transfer of the rheophytic endemic liverwort *Myriocolea irrorata* to *Colura*. *Phytotaxa* 66 (1): 55–64.
<https://doi.org/10.11646/phytotaxa.66.1.9>
- Heinrichs, J., Dong, S., Schäfer-Verwimp, A., Pócs, T., Feldberg, K., Czumaj, A., Schmidt, J., Reitner, A.R., Renner, M.A., Hentschel, J., Stech, M. & Schneider, H. (2013) Molecular phylogeny of the leafy liverwort *Lejeunea* (Porellales): evidence for a neotropical origin, uneven distribution of sexual systems and insufficient taxonomy. *PLoS ONE* 8: 82547.
<https://doi.org/10.1371/journal.pone.0082547>
- Heinrichs, J., Kettunen, E., Lee, G.E., Marzaro, G., Pócs, T., Ragazzi, E., Renner, M.A., Rikkinen, J., Sass-Gyarmati, A. & Schäfer-Verwimp, A. (2015) Lejeuneaceae (Marchantiophyta) from a species-rich taphocoenosis in Miocene Mexican amber, with a review of liverworts fossilised in amber. *Review of Palaeobotany and Palynology* 221: 59–70.
<https://doi.org/10.1016/j.revpalbo.2015.05.007>
- Heinrichs, J., Scheben, A., Bechteler, J., Lee, G.E., Schäfer-Verwimp, A., Hedenäs, L., Singh, H., Pócs, T., Nascimbene, P.C., Peralta, D.F., Renner, M. & Schmidt, A.R. (2016) Crown group Lejeuneaceae and pleurocarpous mosses in Early Eocene (Ypresian) Indian amber. *PLoSone* 11: e0156301.
<https://doi.org/10.1371/journal.pone.0156301>
- Herzog, T. (1942) Drei neue *Ceratolejeunea*-Arten aus der Neotropis. *Revue Bryologie Lichénologie* 13: 20–24.
- Hooker, W.J. (1813) *British Jungermanniae: being a history and description, with colored figures, of each species of the genus, and microscopical analyses of the parts*. Part 11, plates 41–44. Longman, London.
- Hoorn, C., Bogotá-A, G.R., Romero-Baez, M., Lammertsma, E.I., Flantua, S.G.A., Dantas, E.L., Dino, R., do Carmo, D.A. & Chemale, F. (2017) The Amazon at sea: Onset and stages of the Amazon River from a marine record, with species reference to Neogene plant turnover in the drainage basin. *Global and Planetary Change* 153: 51–65.
<https://doi.org/10.1016/j.gloplacha.2017.02.005>
- Hoorn, C., Wesselingh, F.P., ter Steege, H., Bermúdez, M.A., Mora, A., Sevink, J., Sanmartín, I., Sanchez-Meseguer, A., Anderson, C.L., Figueiredo, J.P., Jaramillo, C., Riff, D.D., Negri, F.R., Hooghiemstra, H., Lundberg, J., Stadler, T., Särkinen, T. & Antonelli, A. (2010) Amazonia through time: Andean uplift, climate change, landscape evolution and biodiversity. *Science* 330: 927–931.
<https://doi.org/10.1126/science.1194585>
- Iturralde-Vinent, M.A. & MacPhee, R.D.E. (1996) Age and paleogeographical origin of Dominican amber. *Science* 273: 1850–1852.
<https://doi.org/10.1126/science.273.5283.1850>
- Jack, J.B. & Stephani, F. (1892) Hepaticae Wallisianae. *Hedwigia* 31: 11–27.
- Jaramillo, C., Romero, I., D’Apolito, C., Bayona, G., Duarte, E., Louwye, S., Escobar J., Luque J., Carrillo-Briceño, J.D., Zapata, V., Mora, A., Schouten, S., Zavada, M., Harrington, G., Ortiz, J. & Wesselingh, F.P. (2017) Miocene flooding events of western Amazonia. *Science Advances* 3: e1601693.
<https://doi.org/10.1126/sciadv.1601693>
- Laenen, B., Shaw, B., Schneider, H., Goffinet, B., Paradis, E., Désamoré, A., Heinrichs, J., Villarreal, J.C., Gradstein, S.R., McDaniel, S.F., Long, D.G., Forrest, L.L., Hollingsworth, M.L., Crandall-Stotler, B., Davis, E.C., Engel, J., von Konrat, M., Cooper, E.D., Patiño, C.J., Cox, J., Vanderpoorten, A. & Shaw, A.J. (2014) Extant diversity of bryophytes emerged from successive post-Mesozoic diversification bursts. *Nature Communications* 5: 5134.
<https://doi.org/10.1038/ncomms6134>
- Lanfear, R., Frandsen, P.B., Wright, A.M., Senfeld, T. & Calcott, B. (2016) PartitionFinder 2: New Methods for Selecting Partitioned Models of Evolution for Molecular and Morphological Phylogenetic Analyses. *Molecular Biology and Evolution* 34: 772–773.
<https://doi.org/10.1093/molbev/msw260>
- Libert, M.-A. (1820) Sur un genre nouveau d’hépatiques, *Lejeunia*. *Annales générales des sciences physiques* 6: 372–374.
- Malhi, Y., Aragão, L.E.O.C., Galbraith, D., Huntford, C., Fisher, R., Zelazowski, P., Stich, S., McSweeney, C. & Meir, P. (2009) Exploring the likelihood and mechanism of a climate-change induced dieback of the Amazon rainforest. *Proceedings of the National Academy of Sciences* 106: 20610–20615.
<https://doi.org/10.1073/pnas.0804619106>

- Miller, M.A., Pfeiffer, W. & Schwartz, T. (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees” in Proceedings of the Gateway Computing Environments Workshop (GCE). Available from: <http://www.phylo.org/index.php> (accessed 24 December 2018)
- Piippo, S. (1986) A monograph of the genera *Lepidolejeunea* and *Luteolejeunea* (Lejeuneaceae, Hepaticae). *Acta Botanica Fennici* 132: 1–69.
- Pócs, T. (2002) New or little known epiphyllous liverworts, IX. Two new neotropical *Cololejeunea* species. *Acta Botanica Hungarica* 44: 371–382.
<https://doi.org/10.1556/ABot.44.2002.3-4.13>
- Pócs, T. (2011) East African Bryophytes XXIX. The *Ceratolejeunea* (Lejeuneaceae) species of the Indian Ocean Islands. *Polish botanical journal* 56: 131–153.
- Reiner-Drehwald, M.E. (2011) Studies on Neotropical Lejeuneaceae (Jungermanniopsida). New Synonyms and *Ceratolejeunea temnantha* (Spruce) *comb. nov. Cryptogamie, Bryologie* 32: 95–100.
<https://doi.org/10.7872/cryb.v32.iss1.2011.095>
- Reiner-Drehwald, M.E. & Weis, G. (2001) On *Cephalantholejeunea* (Lejeuneaceae, Hepaticae) from South America, and its placement in the subfamily Ptychanthoideae, tribe Ptychantheae. *Systematic Botany* 26: 699–703. Available from: <https://www.jstor.org/stable/3093851> (accessed 24 December 2018)
- Rambaut, A. (2014) FigTree v1.4.2. Available from: <http://tree.bio.ed.ac.uk/software/figtree/> (accessed 24 December 2018)
- Rambaut, A., Suchard, M.A., Xie, W. & Drummond, A.J. (2014) TRACER v.1.6, Available from: <http://tree.bio.ed.ac.uk/software/tracer> (accessed 24 December 2018)
- Ronquist, F. & Huelsenbeck, J.P. (2003) MrBayes3: Bayesian phylogenetic inference undermixed models. *Bioinformatics* 19: 1572–1574.
<https://doi.org/10.1093/bioinformatics/btg180>
- Sande Lacoste, C.M. van der (1856) Synopsis hepaticarum javanicarum. C. G. van der Post, Amsterdam, pp. 1–112.
- Scheben, A., Bechteler, J., Lee, G.E., Pócs, T., Schäfer-Verwimp, A. & Heinrichs, J. (2016) Multiple transoceanic dispersals and geographical structure in the pantropical leafy liverwort *Ceratolejeunea* (Lejeuneaceae, Porellales). *Journal of Biogeography* 43: 1739–1749.
<https://doi.org/10.1111/jbi.12779>
- Schevock, J.R., Ma, W.-Z. & Akiyama, H. (2017) Diversity of the rheophytic condition in bryophytes: field observations from multiple continents. *Bryophyte Diversity and Evolution* 39: 75–93.
<https://doi.org/10.11646/bde.39.1.12>
- Schiffner, V. (1893) Hepaticae. In: Engler, H.G.A. & Prantl, K. (Eds.) *Die Natürlichen Pflanzenfamilien*, Teil. I, Abt. 3. Engelmann, Leipzig, pp. 1–144.
- Schuster, R.M. (1956) North American Lejeuneaceae. V. Schizostipae: *Ceratolejeunea*. *Journal of the Elisha Mitchell Scientific Society* 72: 292–316.
- Schuster, R.M. (1978) Studies on Venezuelan Hepaticae. II. *Phytologia* 39: 425–432.
<https://doi.org/10.5962/bhl.part.7622>
- Schuster, R.M. (1980) *The Hepaticae and Anthocerotae of North America East of the Hundredth Meridian*. Vol IV. Columbia University Press, New York. 1334 pp.
- Shephard, G.E., Müller, R.D., Liu, L. & Gurnis, M. (2010) Miocene drainage reversal of the Amazon River driven by plate–mantle interaction. *Nature Geoscience* 3: 870–875.
<https://doi.org/10.1038/ngeo1017>
- Söderström, L., Hagborg, A., von Konrat, M., Bartholomew-Began, S., Bell, D., Briscoe, L., Brown, E., Cargill, D.C., da Costa, D.P., Crandall-Stotler, B.J., Cooper, E., Dauphin, G., Engel, J., Feldberg, K., Glenny, D., Gradstein, S.R., He, X., Hentschel, J., Ilkiu-Borges, A.L., Katagiri, T., Konstantinova, N.A., Larrain, J., Long, D., Nebel, M., Pócs, T., Puche, F., Reiner-Drehwald, E., Renner, M., Sass-Gyarmati, A., Schäfer-Verwimp, A., Segarra-Moragues, J., Stotler, R.E., Sukkharak, P., Thiers, B., Uribe, J., Váña, J., Wigginton, M., Zhang, L. & Zhu, R.L. (2016) World checklist of hornworts and liverworts. *PhytoKeys* 59: 1–828s.
<https://doi.org/10.3897/phytokeys.59.6261>
- Solórzano Kraemer, M.M. (2007) Systematic, palaeoecology, and palaeobiogeography of the insect fauna from Mexican amber. *Palaeontographica Abteilung A*, 282: 1–133.
<https://doi.org/10.1127/pala/282/2007/1>
- Spruce, R.M. (1884–1885) Hepaticae Amazonicae et Andinae. *Transactions & Proceedings of the Botanical Society of Edinburgh* 15: i–xi, 1–588.
- Stamatakis, A. (2006) RAxML-VI-HPC: Maximum Likelihood-based Phylogenetic Analyses with Thousands of Taxa and Mixed Models. *Bioinformatics* 22: 2688–2690.

<https://doi.org/10.1093/bioinformatics/btl446>

Stephani, F. (1913) *Species hepaticarum* 5. George & Cie, Genève & Bale, pp. 177–448.

<https://doi.org/10.5962/bhl.title.95494>

Swofford, D. (2002) PAUP*: phylogenetic analysis using parsimony (* and other methods), version 4.0a146. Sinauer, Sunderland, MA.

Thiers, B.M. (1984) Branching in Lejeuneaceae II. Nipponolejeuneoideae, Tuyamaelloideae and Myriocoleoideae. *Lindbergia* 10: 4–8.

<https://www.jstor.org/stable/20149502>

Thiers, B.M. (1988) Morphological adaptation of the Jungermanniales (Hepaticae) to the tropical rainforest habitat. *Journal of the Hattori Botanical Laboratory* 64: 5–14.

Wilson, R., Gradstein, S.R., Schneider, H. & Heinrichs, J. (2007a) Unravelling the phylogeny of Lejeuneaceae (Jungermanniopsida): evidence for four main lineages. *Molecular Phylogeny and Evolution* 43: 270–282.

<https://doi.org/10.1016/j.ympev.2006.10.017>

Wilson, R., Gradstein, S.R., Schneider, H. & Heinrichs, J. (2007b) Steady diversification of derived liverworts under steady Tertiary climatic fluctuations. *Biology Letters* 3: 566–569.

<https://doi.org/10.1098/rsbl.2007.0287>

Yu, Y., Heinrichs, J., Schäfer-Verwimp, A., Zhu, R.-L. & Schneider, H. (2014) Inferring the accumulation of morphological disparity in epiphyllous liverworts. *Organisms Diversity & Evolution* 14: 151–161.

<https://doi.org/10.1007/s13127-014-0166-6>

Zheng, Y. & Wiens, J.J. (2015) Do missing data influence the accuracy of divergence-time estimation with BEAST? *Molecular Phylogenetics and Evolution* 85: 41–49.

<https://doi.org/10.1016/j.ympev.2015.02.002>